(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 147 665**
A1

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 84114536.0

(22) Date of filing: 30.11.84

(51) Int. Cl.⁴: G 01 N 33/50
G 01 N 33/531, C 12 Q 1/68

(30) Priority: 12.12.83 US 560462
19.10.84 US 662858

(43) Date of publication of application:
10.07.85 Bulletin 85/28

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

(71) Applicant: Molecular Diagnostics, Inc.
400 Morgan Lane
West Haven, CT.06516(US)

(72) Inventor: Dattagupta, Nanibhushan
470 Prospect Street
New Haven, Ct 06511(US)

(72) Inventor: Rae, Peter M. M.
71 Ingram Street
Hamden, Ct 06517(US)

(72) Inventor: Knowles, William J.
101 Sleeping Giant Drive
Hamden, Ct 06518(US)

(72) Inventor: Crothers, Donald M.
8 Surrey Drive
Northford, Ct 06472(US)

(74) Representative: Adrian, Albert, Dr. et al,
c/o BAYER AG Konzernverwaltung RP Patentabteilung
D-5090 Leverkusen 1, Bayerwerk(DE)

(54) **Nucleic acid probe, test method and reagent system for detecting a polynucleotide sequence and antibody therefor.**

(57) A nucleic acid detection probe comprising a hybridizable single stranded portion of nucleic acid connected with a non-hybridizable, single or double stranded nucleic acid portion, the non-hybridizable portion preferably including a recognition site for binding by a particular protein. Such recognition site can be a region of singly or doubly stranded nucleic acid specific for a particular nucleic acid binding protein such as lac repressor protein or can be a modified nucleic acid region such as a unique antigenic determinant introduced by interaction of the region with a modifier compound such as an intercalating agent or a platinium-containing ligand. The probe-binding protein can be labeled for ease of detection and in the case of an antigenic determinant binding site can be labeled antibody.

EP 0 147 665 A1

This application relates to a labeled nucleic acid probe suitable for analytical and diagnostic purposes with regard to genetic constitution, and particularly applicable to hybridization assays to detect specific polynucleotide sequences.

The evaluation of nucleic acid hybridizations is usually accomplished by detecting radioactivity introduced into a DNA:DNA or DNA:RNA hybrid via one member of a pair of complementary polynucleotides (the labeled member being designated the probe). Radiolabeling of the probe is effected by in vivo or in vitro polymerization of RNA or DNA under conditions in which precursors are isotopically tagged with ^{3}H, ^{14}C, ^{125}I or ^{32}P, although it is also possible to label polynucleotides postsynthetically using ^{125}I or ^{32}P-ATP. Each kind of radiolabeling has limitations, such as sensitivity of detection, isotope half-life and hazard, and it is highly desirable that probe labeling be accomplished without resort to radioactivity.

Available alternatives are (i) the attachment of haptens such as biotin to nucleic acid precursors, in which case an investigator is required to carry out in vitro polynucleotide syntheses in order to label a probe, then to detect the presence of biotinylated probe in a hybrid through the application of two or more steps; and (ii) the attachment of enzymes to oligonucleotide or polynucleotide probes, in which case, hybrids are detected by their ability to convert a substrate to an optically or chemically distinguishable product. Both of these alternatives to radioactivity involve moderate to substantial changes in the chemical structure of probes, so that qualitative and/or quantative effects on hybridization are a possibility ,if not a reality.

European Patent Application No. 84107248.1 discloses a dual hybridization assay conducted

with a known and an unknown nucleic acid sample and a nucleic acid-containing detection probe. Advantageously, the known sample, the separation probe, is immobilized on a solid support and contacted with the unknown and the instant labeled detection probe. The contact is performed under conditions favorable to hybridization. A portion of the unknown nucleic acid hybridizes with the immobilized probe. If the unknown also contains a nucleotide sequence which is complementary to the nucleotide sequence of the detection probe, a second or dual hybridization will then take place by which the detection probe becomes affixed to the solid support. If the unknown nucleic acid lacks the particular complementary nucleotide sequence, the detection probe will not hybridize therewith. Accordingly, the extent of the second hybridization, as indicated by the extent of labeling, is an indicator of the presence of the particular nucleotide sequence of interest in the unknown.

It then becomes necessary to determine how much of the second hybridization has taken place, i.e., how much of the detection probe is on the immobilized support.

The detection probe can be labeled with various labels which can be detected in systems that measure specific binding activity, fluorescence or enzyme activity. Such labels include radioisotopes, fluorescent radicals, enzymes and haptens. If too many labels are provided as in the case of fluorophores, they may interfere with the second hybridization. On the other hand, if there are too few labels, the assay is less sensitive.

It is, accordingly, an object of the present invention to provide a detection probe (or the probe carrying the labels) which can be used in an assay without the disadvantages of radioactivity and without chemical modification of the probe components which could interfere with hybridization.

It is another object of the invention to provide a means of labeling a probe with a large number of readable labels resulting in relatively high sensitivity, without interfering with hybridization.

These and other objects and advantages are realized in accordance with the present invention pursuant to which there is provided a detection probe comprising a hybridizable single stranded portion of nucleic acid which can hybridize with the unknown, connected with a non-hybridizable single or double stranded nucleic acid portion, the non-hybridizable portion advantageously including a recognition or binding site for a particular protein. If the non-hybridizable portion is double stranded, one of the strands may be continuous, i.e., covalently associated, with the hybridizable portion.

The hybridizable portion of nucleic acid can be any of those described in greater detail with regard to the detection probe of European Patent Application No. 84107248.1, as for example, a nucleotide sequence which is complementary to the genomic sequence responsible for sickle cell anemia.

The nucleic acid of the non-hybridizable portion can be a natural DNA sequence or synthetic oligonucleotide which contains a highly specific binding site or sites for a protein or proteins. A variety of nucleic acid binding site/binding protein pairs can be used in the present invention. One class of useful binding proteins are those known in biological systems to recognize specific polynucleotide sequences such as repressor proteins. Another class are antibodies which can bind to immunogenically altered nucleic acids.

In a preferred embodiment, the non-hybridizable portion can be specific for lactose (hereinafter referred to as lac) repressor protein which binds to an operator locus in the non-hybridizable portion, which operator

must be double stranded, preferably after hybridization, as hybridization might sever the bond between operator and repressor. Accordingly, if the now-immobilized detection probe is contacted with a solution containing lac repressor protein, that protein will be selectively removed from the solution and will bind to the lac operator. Even if the concentration of nonspecific DNA in the hybridized sample is 1000-fold excess, the binding to nonspecific sequences is negligible. In living cells, repressor proteins bind to their corresponding operator sequences to modulate transcription of a gene. When an operator sequence is covalently attached to other sequences, binding of repressor proteins is still specific for the operator.

In another preferred embodiment, the non-hybridizable portion of the probe is chemically and/or physically modified to create a protein recognition site, for example, by interaction of a modifier compound which introduces a unique antigenic determinant into the non-hybridizable portion. Such modifier compounds are exemplified by intercalating agents and platinum-containing ligands. Intercalating agents interact with double stranded nucleic acids by becoming noncovalently inserted between base pairs. Such insertion causes the tertiary structure of the helix to change by unwinding and elongation along the helical axis. The resulting intercalation complex is characterized by newly formed antigenic determinants which are understood to comprise the intercalated compound and the reoriented phosphodiesterase backbones of the respective strands of the duplex. Useful intercalating agents are generally planar, aromatic organic molecules as exemplified by the acridine dyes, e.g., acridine orange, the phenanthridines, e.g., ethidium, the phenazines, furocoumarins, phenothiazines, and quinolines. Essentially any compound which will bind to single or double stranded nucleic acid

to induce an immunogenic change can be used as the modifier compound.

The invention is applicable to all conventional hybridization assay formats. In particular, the unique dectection probe of the present invention can be used in solution and solid-phase hybridization formats, including, in the latter case, formats involving immobilization of either sample or probe nucleic acids and sandwich formats. In general, the present invention provides a method for detecting a particular polynucleotide sequence in a test medium containing single stranded nucleic acids, comprising the step of

(a) combining the test medium with a nucleic acid detection probe comprising at least one hybridizable single stranded base sequence which is substantially complementary to the sequence to be detected and a non-hybridizable double stranded portion having a recognition site for binding by a particularly protein, under conditions favorable to hybridization between the sequence to be detected and the complementary hybridizable sequence in the probe,

b) separating the solid support carrying hybridized probe from unhybridized probe, and

c) adding to the separated solid support carrying hybridized probe, the particular protein which binds the recognition site on the non-hybridizable portion of the probe and determining the protein which becomes bound to the solid support.

In one such assay for the presence of a particular nucleic acid nucleotide sequence in a sample, either the sample or a separation probe is immobilized on a support and, with a detection probe as described hereinabove, is subjected to hybridization, thereby affixing the non-hybridizable portion to the support. A protein is bound to the protein recognition site and thereby to the support. The protein is labeled at any stage, either before or after binding, and finally the

label is assayed.

In another assay, after binding the protein and separating the support from the balance of the material, the support is treated in order to dissociate the protein from the hybridized detection probe and the dissociated protein is then assayed as by reading a label thereon, the label having been applied at any prior stage.

The invention is further described with reference to the accompanying drawing which is a schematic flow sheet of a hybridization and assay in accordance with the present invention.

Referring now more particularly to the drawing, the unknown DNA (to be tested) is processed as by digestion with a restriction enzyme, electrophoretic separation, southern transfer and/or simple denaturation. If not already immobilized, the DNA is then adsorbed on to a solid support (e.g., nitrocellulose paper) directly or by hybridization to a separation probe. The immobilized DNA is hybridized with a known probe. The known probe (P) has two regions. The region ps is single stranded and complementary to a specific gene to be detected and the region pd is a piece of double or single stranded, non-homologous DNA which carries the labels by which the labeling reaction will be detected. The pd region can be a specific sequence of double stranded DNA which binds a specific protein. For example, the double stranded DNA can be the lac promoter/operator sequence and then the protein is lac repressor. The pd region can also be a binding site for a specific antibody. The pd region can also be a specific single stranded, immunogenic polynucleotide sequence or poly [d(G-C)] which, when treated with high salt, changes its structure and becomes immunogenic in the Z form. The pd portion can also be modified with intercalating agents or platinum-containing DNA binding ligands to produce immunogenic sites.

If pd is the lac promoter/operator sequence, pd will bind lac repressor protein after the hybridization. The protein can then be assayed by an antibody or by direct labeling. The double stranded pd portion can also be modified with hapten, e.g., biotin. Then the biotinylated hybrid can be detected in a known manner. The pd portion can also be modified with a number of fluorophores and can be assayed directly.

In a specific embodiment involving a lac operator-repressor system, the foregoing process involves at least the following four steps:

Step I: Grow bacteria and isolate lac repressor protein;

Step II: Covalently couple the detection probe to lac operator DNA and clone the adduct to have a large quantity of sample;

Step III: Prepare lac repressor - FITC or lac repressor- β-galactosidase adduct or anti-lac repressor antibody;

Step IV: Hybridization and detection of lac operator via label on the lac repressor.

Thereafter, the amount of bound lac repressor protein can be assayed in various ways. For example, antibodies thereto can be contacted with the bound lac repressor and protein A conjugated with an enzyme can be bound to the antibodies. The amount of bound enzyme can then be determined by the enzyme's catalytic reaction of its substrate in a conventional manner.

The amount of enzyme indicates the amount of lac repressor which, in turn, indicates the amount of hybridization which occurred earlier. Alternatively, the lac repressor protein can be fluorescently labeled or labeled with enzyme and read in a conventional manner.

The double stranded region of the detection probe can also be specific for galactose repressor proteins, lambda repressor protein, catabolite gene activator protein, CAP, Cro protein and the like. The foregoing

description for assay of bound lac repressor protein applies equally to assay for the presence of these proteins. Such proteins can be purified from strains of Escherichia coli. The DNA sequences to which these proteins bind have been identified and isolated using recombinant DNA technology. The segment of E. coli DNA that contains the lac repressor binding site (the lac promoter operator region) is transferred to recombinant plasmids that include segments of human DNA, such as portions of the gene encoding hemoglobin. These can be used without further genetic engineering to test for a number of hemoglobinopathies, such as some thalassemias and sickle-cell hemoglobinemia. Alternatively, in the dual hybridization scheme, two plasmids are used to determine if a sample of DNA from a human subject contains the genetic condition responsible for sickle cell hemoglobinemia. One plasmid is designated the separation probe. It contains DNA that is one flank of the dimorphic restriction enzyme cleavage site; it is immobilized as single stranded molecules on a solid support, and it is unlabeled. The second plasmid is designated the detection probe. It contains DNA that is the other flank of the dimorphic restriction site, and it has also been engineered to contain a segment of E. coli DNA that contains the lac promoter/operator region. Through the use of appropriate enzymes, the detector plasmid is made partially single stranded to the extent that β-globin gene sequences are available for hybridization while lac repressor recognition sites remain double stranded and available for protein binding.

Read out involving lac repressor protein provides highly specific recognition of the presence of the detection probe. It also opens a new set of possibilities for solution phase read out, because it is possible to release the repressor-antibody complexes from

the operator DNA by addition of a β-galactoside, such as isopropylthio-galactoside. This allows automated batch or flow system processing.

In the foregoing description, the double stranded nucleic acid sequence contained a protein recognition site from the outset. However, if it did not contain such a site initially, it is possible to modify the DNA to create protein or antibody recognition sites for ease of reaction and detection.

Such modification can be effected by contact with intercalating agents, such as furocoumarins, e.g., angelicins, psoralens, etc., as described more fully in European Patent Application No. 84107624.3. Platinum-containing ligands can be similarly employed. The reagents render the non-hybridizable nucleic acid portion recognizable by protein. If the non-hybridizable portion is rendered immunogenic, such protein can be an antibody, i.e., an immunoglobulin, for example, a monoclonal antibody. The antibody can be bound to the non-hybridizable portion in an amount corresponding to the amount of furocoumarin creating the protein recognition sites. Antibody recognition sites can also be created when pd contains poly [d(G-C)] sequences, and the probe is exposed to high salt concentration.

In the case where the modifier compound is an intercalating agent, such compound preferably is a low molecular weight, planer, usually aromatic but sometimes polycyclic, molecule capable of binding with double stranded nucleic acids, e.g., DNA/DNA, DNA/RNA, or RNA/RNA duplexes, usually by insertion between base pairs. The primary binding mechanism will usually be noncovalent, with covalent binding occurring as a second

step where the intercalator has reactive or activatable chemical groups which will form covalent bonds with neighboring chemical groups on one or both of the intercalated duplex strands. The result of intercalation is the spreading of adjacent base pairs to about twice their normal separation distance, leading to an increase in molecular length of the duplex. Further, unwinding of the double helix of about 12 to 36 degrees must occur in order to accommodate the intercalator. General reviews and further information can be obtained from Lerman, J. Mol. Biol. 3:18(1961); Bloomfield et al, "Physical Chemistry of Nucleic Acids", Chapter 7, pp. 429-476, Harper and Rowe, NY(1974); Waring, Nature 219:1320 (1968); Hartmann et al, Angew. Chem., Engl. Ed. 7:693(1968); Lippard, Accts. Chem. Res. 11:211(1978); Wilson, Intercalation Chemistry (1982),445; and Berman et al, Ann. Rev. Biophys. Bioeng. 10:87(1981).

A wide variety of intercalating agents can be used in the present invention. Some classes of these agents and examples of specific compounds are given in the following table:

Intercalator Classes and Representative Compounds	Literature References
A. Acridine dyes	Lerman, J., supra; Bloomfield et al, supra;
proflavin, acridine orange, quinacrine, acriflavine	Miller et al, Biopolymers 19:2091(1980)
B. Phenanthridines	Bloomfield et al, supra; Miller et al, supra
ethidium coralyne	Wilson et al, J. Med. Chem. 19:1261(1976)
ellipticine, ellipticine cation and derivatives	Festy et al, FEBS Letters 17:321(1971); Kohn et al, Cancer Res. 35:71(1976); LePecq et al, PNAS (USA)71: 5078(1974); Pelaprat et

		Real, J. Med. Chem. 23:1330(1980)
C.	Phenazines 5-methylphenazine cation	Bloomfield et al, supra
D.	Phenothiazines chlopramazine	ibid
E.	Quinolines chloroquine quinine	ibid
F.	Aflatoxin	ibid
G.	Polycyclic hydrocarbons and their oxirane derivatives	ibid
	3,4-benzopyrene, benzopyrene diol epoxide, 1-pyrenyl-oxirane	Yang et al, Biochem. Biophys. Res. Comm. 82:929(1978)
	benzanthracene-5,6-oxide	Amea et al, Science 176:47(1972)
H.	Actinomycens actinomycin D	Bloomfield et al, supra
I.	Anthracyclinones β-rhodomycin A daunamycin	ibid
J.	Thiaxanthenones miracil D	ibid
K.	Anthramycin	ibid
L.	Mitomycin	Ogawa et al, Nucl. Acids Res., Spec. Publ. 3:79(1977); Akhtar et al, Can. J. Chem. 53:2891(1975)
M.	Platinum Complexes	Lippard, Accts. Chem. Res. 11:211(1978)
N.	Polyintercalators echinomycin	Waring et al, Nature 252:653(1974); Wakelin, Biochem. J. 157:721(1976)
	quinomycin	Lee et al, Biochem. J.

	triostin BBM928A tandem	173:115(1978); Huang et al, Biochem. 19: 5537(1980): Viswamitra et al, Nature 289: 817(1981)
	diacridines	LePecq et al, PNAS (USA)72:2915(1975): Carrellakis et al, Biochim. Biophys. Acta 418:277(1976); Wakelin et al, Biochem 17:5057(1978); Wakelin et al, FEBS Lett. 104:261(1979); Capelle et al, Biochem. 18:3354(1979); Wright et al, Biochem. 19:5825(1980); Bernier et al, Biochem. J. 199:479 (1981); King et al, Biochem. 21:4982(1982)
	ethidium dimer	Gaugain et al, Biochem. 17:5078(1978); Kuhlman et al, Nucl. Acids Res. 5:2629(1978); Marlcovits et al, Anal. Biochem. 94:259(1979): Dervan et al, JACS 100:1968(1978); ibid 101:3664(1979).
	ellipticene dimers and analogs	Debarre et al, Compt. Rend. Ser. D. 284: 81(1977); Pelaprat et al, J. Med. Chem. 23:1336(1980)
	heterodimers	Cain et al, J. Med. Chem. 21:658(1978); Gaugain et al, Biochem. 17:5078(1978)
	trimers	Hansen et al, JCS Chem. Comm. 162(1983); Atnell et al, JACS 105:2913(1983)
O.	Norphillin A	Loun et al, JACS 104: 3213(1982)
P.	Fluorenes and fluorenones	Bloomfield et al, supra

fluorenodiamines	[illegible]owski et al, Wiss. Beitr.-Martin-Luther-Univ. Halle Wittenberg, 11(1981)
Q. Furocoumarins	
angelicin	Venema et al, MGG, Mol. Gen. Genet. 179;1 (980)
4,5'-dimethylangelicin	Vedaldi et al, Chem.-Biol. Interact. 36: 275(1981)
psoralen	Marciani et al, Z. Naturforsch B 27(2): 196(1972)
8-methoxypsoralen	Belognzov et al, Mutat. Res. 84:11(1981); Scott et al, Photochem. Photobiol. 34:63(1981)
5-aminomethyl-8-methoxypsoralen	Hansen et al, Tet. Lett. 22:1847(1981)
4,5,8-trimethylpsoralen	Ben-Hur et al, Biochim. Biophys. Acta 331:181(1973)
4'-aminomethyl-4,5,8-trimethylpsoralen	Issacs et al, Biochem. 16:1058(1977)
xanthotoxin	Hradecma et al, Acta Virol. (Engl. Ed.) 26:305(1982)
khellin	Beaumont et al, Biochim. Biophys. Acta 608:1829(1980)
R. Benzodipyrones	Murx et al, J. Het. Chem. 12:417(1975); Horter et al, Photochem. Photobiol. 20: 407(1974)
S. Monostral Fast Blue	Juarranz et al, Acta Histochem. 70:130 (1982)

Where desirable or particularly advantageous, the intercalating agent can be chemically linked, e.g., by covalent bonds, to one or both of the complementary strands in the intercalation complex. Essentially any available method can be used to accomplish such linkage. Preferably, the linkage is formed by effecting intercalation with a photoreactive intercalator, followed by the photochemical linking reaction. A particularly useful method involves the use of azido-intercalators. The reactive nitrenes are readily generated at long wavelength ultraviolet or visible light and the nitrenes of arylazides prefer insertion reactions over their rearrangement products [see White et al, Methods in Enzymol. 46:644(1977)]. Representative azidointercalators are 3-azidoacridine, 9-azidoacridine, ethidium monoazide, ethidium diazide, ethidium dimer azide (Mitchell et al, JACS 104:4265(1982)], 4-azido-7-chloro-quinoline, and 2-azidofluorene. Other useful photoreactable intercalators are the furocoumarins which form [2+2] cycloadducts with pyrimidine residues. Alkylating agents can also be used such as bis-chloroethylamines and epoxides or aziridines, e.g., aflatoxins, polycyclic hydrocarbon epoxides, mitomycin, and norphillin A.

Alternatively, the protein recognition site can be on a segment of the non-hybridizable portion other than nucleic acid per se. For example, the nucleic acid of the non-hybridizable portion can be linked by a member such as a furocoumarin to a chemical group such as biotin, the biotin constituting the protein recognition site.

The biotin can be assayed in a conventional manner, for example, with avidin or an anti-hapten antibody. The furocoumarin may be linked to a fluorophore, the fluorophore thereafter being assayed for fluorescence.

Labeling with the aforementioned proteins can be performed either before or after modification of the double stranded nucleic acid, preferably after.

Salts may also be used as a means of modifying the non-hybridizable portion to render it protein recognizable (e.g., poly [d(G-C)] or poly [d(G-^{me}C)] changes to Z-form). Suitable salts include sodium chloride, other alkali and alkaline earth metal soluble salts of mineral acids, spermine or spermidines, advantageously in concentrations of at least about 1% by weight. Advantageously, the solvent is water. Both the salt-modified nucleic acid and the furocouramin modified nucleic acid will be antigenic, e.g., will be capable of binding a specific antibody which can be assayed in conventional manner. For example, as hereinabove, the protein A can be conjugated with an enzyme which functions as the label in subsequent assay.

The probe will comprise at least one single stranded base sequence substantially complementary to or homologous with the sequence to be detected. However, such base sequence need not be a single continuous polynucleotide segment, but can be comprised of two or more individual segments interrupted by nonhomologous sequences. These nonhomologous sequences can be linear, or they can be self-complementary and form hairpin loops. In addition, the homologous region of the probe can be flanked at the 3'- and 5'- termini by nonhomologous sequences, such as those comprising the DNA or RNA of a vector into which the homologous sequence had been inserted for propagation. In either instance, the probe as presented as an analytical reagent will exhibit detectable hybridization at one or more points which sample nucleic acids of interet. Linear or circular single stranded polynucleotides can be used as the probe element, with major or minor portions being duplexed with a complementary polynucleotide strand or strands, provided that the critical homologous segment or segments

are in single stranded form and available for hybridization with sample DNA or RNA. Particularly preferred will be linear or circular probes wherein the homologous probe sequence is in essentially only single stranded form [see particularly, Hu and Messing, Gene 17:271-277(1982)].

The binding of the particular protein reagent to the hybridization product in the present method can be detected by any convenient technique. Advantageously, the binding protein will itself be labeled with a detectable chemical group. Such detectable chemical group can be any material having a detectable physical or chemical property. Such materials have been well-developed in the field of immunoassays and in general most any label useful in such methods can be applied to the present invention. Particularly useful are enzymatically active groups, such as enzymes (see Clin. Chem.(1976)22:1243), enzyme substrates (see British Pat. Spec. 1,548,741), coenzymes (see U.S. Pat. Nos. 4,230,797 and 4,238,565), and enzyme inhibitors (see U.S. Pat No. 4,134,792); fluorescers (see Clin. Chem.(1979)25:353); chromophores; luminescers such as chemiluminescers and bioluminescers (see Clin. Chem.(1979)25:512, and ibid, 1531); specifically bindable ligands; and radioisotopes such as ^{3}H, ^{35}S, ^{32}P, ^{125}I, and ^{14}C. Such labels and labeling pairs are detected on the basis of their own physical properties (e.g., fluorescers, chromophores and radioisotopes) or their reactive or binding properties (e.g., enzymes, substrates, coenzymes and inhibitors). For example, a cofactor-labeled binding protein can be detected by adding the enzyme for which the label is a cofactor and a substrate for the enzyme. A hapten or ligand (e.g., biotin) labeled binding protein can be detected by adding an antibody to the hapten or a protein (e.g., avidin) which binds the ligand, tagged with a detectable molecule. Such detectable molecule can be some molecule

with a measurable physical property (e.g., fluorescence or absorbance) or a participant in an enzyme reaction (e.g., see above list). For example, one can use an enzyme which acts upon a substrate to generate a product with a measurable physical property. Examples of the latter include, but are not limited to, β-galactosidase, alkaline phosphatase and peroxidase. For in situ hybridization studies, ideally the final product is water insoluble. Other labeling schemes will be evident to one of ordinary skill in the art.

Alternatively, the binding protein can be detected based on a native property such as its own antigenicity. A labeled anti-(binding protein)antibody will bind to the primary protein reagent where the label for the antibody is any conventional label as above. Further, where the binding protein is an antibody, it can be detected by complement fixation or the use of labeled protein A, as well as other techniques known in the art for detecting antibodies.

The label will be linked to the binding protein by direct chemical linkage such as involving covalent bonds, or by indirect linkage such as by incorporation of the label in a microcapsule or liposome which is in turn linked to the binding protein. Labeling techniques are well-known in the art and any convenient method can be used in the present invention.

When the binding protein is an antibody, such reagent can consist of whole antibodies, antibody fragments, polyfunctional antibody aggregates, or in general any substance comprising one or more specific binding sites from an antibody. When in the form of whole antibody, it can belong to any of the classes and subclasses of known immunoglobulines, e.g., IgG, IgM, and so forth. Any fragment of any such antibody which retains specific binding affinity for the probe recognition site can also be employed, for instance, the fragments of IgG conventionally known as Fab, F(ab'), and

$F(ab')_2$. In addition, aggregates, polymers and conjugates of immunoglobulins or their fragments can be used where appropriate.

The immunoglobulin source for the antibody reagent can be obtained in any available manner such as conventional antiserum and monoclonal techniques. Antiserum can be obtained by well-established techniques involving immunization of an animal, such as a mouse, rabbit, guinea pig or goat, with an appropriate immunogen. The immunogen will usually comprise an ionic complex between a cationic protein or protein derivative (e.g., methylated bovine serum albumin) and the modified nucleic acid. Alternatively, the modified nucleic acid can be covalently coupled to a carrier protein. The immunoglobulins can also be obtained by somatic cell hybridization technique, such resulting in what are commonly referred to as monoclonal antibodies. The immunogen used for primary injections leading to hybridoma formation will be as described above.

The test sample to be assayed can be any medium of interest, and will usually be a liquid sample of medical, veterinary, environmental, nutritional, or industrial significance. Human and animal specimens and body fluids particularly can be assayed by the present method, including urine, blood (serum or plasma), milk, cerebrospinal fluid, sputum, fecal matter, lung aspirates, throat swabs, genital swabs and exudates, rectal swabs, and nasopharyngal aspirates. Where the test sample obtained from the patient or other source to be tested contains principally double stranded nucleic acids, such as contained in cells, the sample will be treated to denature the nucleic acids, and if necessary first to release nucleic acids from cells. Denaturation of nucleic acids is preferably accomplished by heating in boiling water or alkali treatment (e.g., 0.1 N sodium hydroxide), which if desired, can simultaneously be used to lyse cells. Also, release of nucleic acids can, for

example, be obtained by mechanical disruption (freeze/thaw, abrasion, sonication), physical/chemical disruption (detergents such as Triton, Tween, sodium dodecylsulfate, alkali treatment, osmotic shock, or heat), or enzymatic lysis (lysozyme, proteinase K, pepsin). The resulting test medium will contain nucleic acids in single stranded form which can then be assayed according to the present hybridization method.

As is known in the art, various hybridization conditions can be employed in the assay. Typically, hybridization will proceed at slightly elevated temperatures, e.g., between about 35 and 70½C and usually around 65½C, in a solution comprising buffer at pH between about 6 and 8 and with appropriate ionic strength (e.g., 2XSSC where 1XSSC = 0.15M sodium chloride and 0.015M sodium citrate, pH 7.0), protein such as bovine serum albumin, Ficoll (a trademark identifying a copolymer of sucrose and epichlorohydrin sold by Pharmacia Fine Chemicals, Piscataway, NJ), polyvinylpyrrolidone, and a denatured foreign DNA such as from calf thymus or salmon sperm. The degree of complementarity between the sample and probe strands required for hybridization to occur depends on the stringency of the conditions. The extent and specificity of hybridization is affected by the following principal conditions:

1. The purity of the nucleic acid preparation.

2. Base composition of the probe - G-C base pairs will exhibit greater thermal stability than A-T base pairs. Thus, hybridizations involving higher G-C content will be stable at higher temperatures.

3. Length of homologous base sequence - Any short sequence of bases (e.g., less than 6 bases), has a high degree of probability of being present in many nucleic acids. Thus, little or no specificity can be attained in hybridizations involving such short sequences. The

present homologous probe sequence will be at least 10 bases, usually 20 bases or more, and preferably greater than 100 bases. From a practical standpoint, the homologous probe sequence will often be between 300-1000 nucleotides.

4. Ionic strength - The rate of reannealing increases as the ionic strengthh of the incubation solution increases. Thermal stability of hybrids also increases.

5. Incubation temperature - Optimal reannealing occurs at a temperature about 25-30°C below the melting temperature (Tm) for a given duplex. Incubation at temperatures significantly below the optimum allows less related base sequences to hybridize.

6. Nucleic acid concentration and incubation time - Normally, to drive the reaction towards hybridization, one of the hybridizable sample nucleic acid will be present in excess, usually 100 fold excess or greater.

7. Denaturing reagents - The presence of hydrogen bond disrupting agents such as formamide and urea increases the stringency of hybridization.

8. Incubation time - The longer the incubation time the more complete will be the hybridization.

9. Volume exclusion agents - The presence of these agents, as exemplified by dextran and dextran sulfate, are thought to effectively increase the concentration of the hybridizing elements thereby increasing the rate of resulting hybridization.

Practice of the present analytical method is not limited to any particular hybridization format. Any conventional hybridization technique can be used. As improvements are made and as conceptually new formats are developed, such can be readily applied to carrying out the present method. Conventional hybridization formats which are particularly useful include those wherein the sample nucleic acids is immobilized on a solid support (solid-phase hybridization) and those wherein the

polynucleotide species are all in solution (solution hybridization).

In solid-phase hybridization formats, sample polynucleotides are fixed in an appropriate manner in their single stranded form to a solid support. Useful solid support are well known in the art and include those which bind nucleic acids either covalently or non-covalently. Noncovalent supports which are generally understood to involve hydrophobic bonding include naturally occurring and synthetic polymeric materials, such as nitrocellulose, derivatized nylon, and fluorinated polyhydrocarbons, in a variety of forms such as filters or solid sheets. Covalent binding supports are also useful and comprise materials having chemically reactive groups or groups, such as dichlorotriazine, diazobenzyloxymethyl, and the like, which can be activated for binding to polynucleotides.

A typical solid-phase hybridization technique begins with immobilization of sample nucleic acids onto the support in single stranded form. This initial step essentially prevents reannealing of complementary strands from the sample and can be used as a means for concentrating sample material on the support for enhanced detectability. The polynucleotide probe is then contacted with the support and hybridization detected as described herein. The solid support provides a convenient means for separating labeled reagent associated with hybridized probe from that which remains unassociated.

Another method of interest is the sandwich hybridization technique wherein one of two mutually exclusive fragments of the homologous sequence of the probe is immobilized and the other is labeled. The presence of the polynucleotide sequence of interest results in dual hybridization to the immobilized and labeled probe segments, again with the same ultimate measurement of support-associated intercalation

complexes. See Methods in Enzymology 65:468(1980) and Gene 21:77-85(1983) for further details.

The invention also extends to assays involving detection probes wherein the non-hybridizable portion has been modified to attach a protein recognition site, as with a furocoumarin, as a link between the non-hybridizable double or single strand component and the protein recognition site, which can be a hapten or ligand. An immobilized separation probe or test sample is subjected to hybridizing conditions in the presence of a detection probe so modified to bind a protein and carrying a label, and the label is assayed. Alternatively, the protein may constitute an antibody and the assayed immunologically in conventional manner, without formal labeling of the protein. As another alternative, if a hapten or ligand is at the the protein recognition site, its presence can be assayed. The furocoumarin can also link a fluorophore and the fluorophore utilized as the assayable element.

The detection probes made and used as described above exhibit greater sensitivity than heretofore by virtue of the far greater number of labels per single stranded nucleic acid probe molecule than is possible with directly-labeling the probe molecules.

The present invention additionally provides a reagent system, i.e., reagent combination or means, comprising all of the essential elements required to conduct a desired assay method. The reagent system is presented in a commercially packaged form, as a composition or admixture where the compatability of the reagents will allow, in a test device configuration, or more usually as a test kit, i.e., a packaged combination of one or more containers, devices, or the like holding the necessary reagents, and usually including written instructions for the performance of assays. Reagent

systems of the present invention include all configurations and compositions for performing the various hybridization formats described herein.

In all cases, the reagent system will comprise (1) a probe as described herein and (2) the binding protein reagent, preferably labeled with a detectable chemical group also as described herein. The system can additionally comprise a solid support for immobilizing single stranded nucleic acids from the test medium. For a sandwich format, a second, separation, probe as described above is included in the system. A test kit form of the system can additionally include ancillary chemicals such as the components of the hybridization solution and denaturation agents capable of converting double stranded nucleic acids in a test sample into single stranded form. Preferably, there is included a chemical lysing and denaturing agent, e.g., alkali, for treating the sample to release single stranded nucleic acid therefrom.

The invention will be further described with reference to the accompanying examples wherein all parts are by weight unless otherwise expressed.

EXAMPLE 1

Step I - Isolating lac repressor protein

E. coli strain BMH 461:

Δ (lac pro) (λ $C_1$857t68d lac $i^q z^+ y^-$)/(F' lac $i^q z^+ y^- pro^+$), developed by Muller-Hill et al., carries a thermally inducible lambda lysogen with a *lac* repressor gene and it overproduces the protein 1000-fold compared to the wild type strain. (Other *E. coli* strains can also be used to isolate the protein). The strain is grown substantially as described by Muller-Hill et al. and Platt et al. [Muller-Hill et al., *Proc. Natl. Acad. Sci.*, 59, 1259 (1968); Platt et al., in *Exp. in Mol*

Genetics (ed., J. Miller) CSH, pp. 363-393 (1972)], as follows:

The cells are grown in a medium containing 3% Bactotryptone, 2% Bacto yeast extract (both from Difco) and 0.5% sodium chloride at 32°C to an OD 550 of 3. Then the temperature is raised to 44°C for 20 minutes to effect for 5 hours. The cells are collected from the culture by centrifugation at 6000 rpm and stored frozen at -80°C.

100 gms of cells are thawed and blended in a Waring blender and the supernatant after centrifugation is made up to 100 ml with a buffer comprising 0.2 M Tris HCl, PH 6.9, 0.2 M KCl, 10 mM mg acetate, 0.1 mM dithiothreitol, 5% (^{v}/v) glycerol) and precipitated by adding 0.23 g/ml ammonium sulfate. The precipitate is collected by centrifugation at 10,000 rpm and redissolved in 5 ml of the foregoing buffer and desalted by exhaustive dialysis against a buffer solution comprising 0.12 M potassium phosphate (PH7.4) 0.1 mM dithiothreitol, 5% (^{v}/v) glycerol; 2% (^{v}/v) dimethyl sulfate.

The lac repressor protein eluate is finally purified on a phosphocellulose column using phosphate buffer as above, with a linear gradient of 0.12 to 0.24 potassium phosphate.

The purity of the lac repressor-containing fraction is checked by SDS-polyacrylamide gel electrophoresis. The activity of the lac repressor protein is measured by its ability to bind operator-containing DNA in a known manner. The protein can be stored at -80°C until use.

Step II - Covalently coupling detection probe to lac operator DNA

Preparation of a plasmid having both multiple copies of the lac repressor protein binding site (lac operator) and a portion of the β-hemoglobin gene, pursuant to

Molecular Cloning, Maniatis et al., Cold Spring Harbor Laboratory, 1982.

1. pHW104 is a derivative of pBR322 that has 4-5 copies of the 203 bp Hae III segment of the lac operon that contains the lac repressor binding site. The segment is tailed with Eco RI linkers, and tandem copies are inserted into the $Ap^R Tc^S$ vector pHW1 (a derivative of PBR322 prepared by Hae II digestion to lack the sequence 236 to 2352) at the Eco Ri site.

2. pSS737 is a derivative of pBR322 that has the 737 bp Alu I segment of the human ß-globin gene that contains about 0.5 kb of the gene and about 0.25 kb of upstream flanking sequence. The segment is tailed with Eco RI linkers and inserted into the Eco Ri site of pBR322.

The procedure for putting the lac repressor binding sites and the segment of the ß-globin gene in the single plasmid as in 1 and 2 above, is as follows:

a. Linearize pHW104 with Hind III; treat with alkaline phosphatase to prevent recircularization in step c.

b. Digest pSS737 with Hind III plus Fnu DII; collect the greater than 0.76 kb segment from a preparative agarose gel.

c. Ligate the products of steps a and b, then fill in free Hind III ends using the Klenow fragment of DNA polymerase and deoxyribnucleotide triphosphates.

d. Blunt-end ligate (c) molecules to make circular plasmids, then transform E. coli cells to ampicillin resistance.

e. Collect a number of Ap^R colonies and grow cells for the minilysate production of small amounts of plasmid.

f. Check the plasmids for composition by restriction enzyme digestion. The desired plasmid has:

i. a single Hind III site;

ii. Eco RI segments of 2.2, 0.74 and 0.21 kb;

iii. digestibility by Mst II;

iv. Desirably a Cla I segment of about 0.75 kb,

depending on the orientation of the globin gene insert.

The separation and detection probe for dual hybridization analysis of sickle cell defect are disclosed in detail in Application Serial No. 511,063, filed July 5, 1983, now pending.

3. Use of a plasmid having both multiple copies of the lac repressor binding site and a portion of the β-hemoglobin gene as a hybridization probe:

For the plasmid to be useful probe for the detection of β-globin gene sequence in a simple of DNA, the globin gene portion of the plasmid must be single stranded so that in a subsequent test it can hybridize to a sample of denatured DNA, and the lac operator region must be double stranded to allow binding of the lac repressor protein.

To achieve this, the plasmid product of (2) is linearized using Hind III, then is subjected to a controlled digestion by exonuclease III (λ exonuclease or T_4DNA polymerase can be similarly employed). Such treatment makes most or all of the globin gene portion single stranded, leaving most of the rest of the plasmid, including the copies of the lac operator region double stranded.

Alternatively, pairs of pEMBL plasmids (available from the European Molecular Biology Laboratories, Heidelberg) can be used. These plasmids contain a portion of the F_1 phage genome, so that they behave like phage M13 in producing single stranded DNA molecules. Unlike with M13, however, it is possible with

pEMBL to collect both complementary strands of a plasmid in pure form simply by having the F_1 portion of the pEMBL genome in different orientation in two strains; it is the orientation of the F_1 genes in the plasmid that determines which of the two strands of the plasmid DNA will be secreted from infected bacteria as single stranded DNA phage.

For example, one plasmid, pEMBL8(+), is engineered to contain tandem copies of the lac repressor binding site plus a portion of the β-hemoglobin gene; another plasmid, pEMBL8(-), contains just the tandem copies of the lac repressor binding site. The single stranded DNA of pEMBL8(+) is hybridized to a sample of unknown DNA, and contact is made through sequence homology between the globin gene portion of the probe and complementary sequences in the sample. The lac operator portion of the probe is made double stranded for lac repressor binding by the annealing of pEMBL8(-) to the pEMBL8(+)-sample DNA complex.

It is possible to carry out such reactions with the replicative (but not the single stranded phage) form of M13 as well as with any plasmid DNA, but one has either to separate the complementary strands, or take considerable loss in hybridization efficiency by having both strands of a plasmid present in a hybridization mixture, where they can undesirably self-anneal.

Step III - (a) Labeling of the protein with fluorescein

Fluoresceinisothiocyanate (FITC) is dissolved in ethanol (5 mg solid/ml). To 2 ml of a 5 mg/ml protein solution from (I), 0.5 ml carbonate buffer (1 M $NaHCO_3$-Na_2CO_3 buffer pH 9) is added, followed by 50 µl FITC solution. The mixture is shaken well and the free FITC is chromatographically separated from the bound molecules on a Sephadex G50 column using a buffer comprising 10 mM Tris, 1 mM EDTA, 50 mM KCl, pH 7.4. The

labeled protein is collected in the void volume.

(b) Labeling with β-galactosidase enzyme

Lac repressor protein from I and β-galactosidase (1:1 molar ratio) (alkaline phosphatase, horseradish peroxidase react similarly) are mixed in phosphate buffer and glutaraldehyde is added to final concentration of 0.2%. Reaction is allowed to proceed for 4 hours. The protein mixture is dialyzed against the same Tris EDTA buffer as in (a).

STEP IV - Hybridization and detection via labels on the lac repressor

Hybridization is done by fixing the separation probe to a solid support according to the example of Application Serial No. 511,063, supra, using as the detection probe that probe produced in II which carries the nonhomologous DNA which is the lac repressor protein binding site. After hybridization, the solid support is washed with BSA solution 1% ω/v in Tris-EDTA buffer as in Example 1, Step III(a), and then lac repressor, labeled as in III(a), (b) or (c), is added. The bound repressor is assayed optically (in the example of fluorescein labeled repressor) or enzymatically (in the example of enzyme labeled repressor).

EXAMPLE 2

Hybridized probe of Example 1 carrying the lac repressor protein can be assayed immunochemically as follows:

a) Purified lac repressor protein from Example 1 is mixed 1:1 with Freund's complete adjuvant and injected into mice (25 µg protein into both hind foot pads) or rabbits (500 µg subcutaneously). One month

later the polyclonal antibody response is titered and the antiserum from animals with strong responses is collected and used for the immunoassays.

b) To the hybrid complex containing the lac repressor protein of Example 1, specific dilutions of the antiserum of (a) are incubated for 1 hour at room temperature. Unbound antibodies are washed 3 times with a buffer solution comprising 5 mM NaH_2PO_4, 150 mM NaCl (pH 7.4) and 0.04% Triton X-100. Protein A covalently coupled to horseradish peroxidase (Sigma Chemical Co. p 8651) diluted 1:8000 in PBS as above, is incubated with the hybridization complex of Example 1 for 30 minutes at room temperature and washed 3 times with the aforementioned buffer. The substrate o-phenylenediamine in citrate buffer containing H_2O_2, pH 5.6, is added and the enzymatic reaction product is measured at 492 nm. The amount of bound repressor is determined by comparison to standard quantitation curves.

EXAMPLE 3

The double stranded portion of the detection probe of Example 1 can be modified to bind a specific antibody as follows:

The detection probe is dissolved in 10 mM Tris 1 mM EDTA buffer and mixed with biotin-psoralen adduct as described in Example 1 of Application Serial No. 513,932, supra. The mixture is irradiated with 360 nm light at room temperature for 40 minutes. After the reaction, the sample is dialyzed against the hybridization buffer of Example 1, to exclude unreacted biotin-psoralen adduct.

The biotin-containing detection probe is then hybridized as in Step IV and the hybrid is assayed for the presence of biotin in known manner employing FITC-labelled avidin.

EXAMPLE 4

Introduction of an antigenic site into double stranded nucleic acid by intercalation.

Covalent ethidium-DNA complexes are prepared as follows: About 250 mg of salmon sperm DNA (Sigma Chemical Co., St. Louis, MO) is dissolved in 40 ml of 50 nM $ZnCl_2$ and sheared by five passages through a 23 gauge needle. The sheared DNA is placed in a 250 ml flask and diluted with an additional 160 ml of buffer. One hundred forty-five microliters (145 µl) of S_1-nuclease, 200,000 units per ml (Pharmacia P-L Biochemicals, Piscataway, NJ), is added and the mixture is incubated at 37°C for 50 minutes.

Then the reaction mixture is extracted twice with phenol:chloroform, once with chloroform and the DNA is precipitated twice with ethanol [Maniatis et al (1982) "Molecular Cloning", Cold Spring Harbor Laboratory, Cold Spring Harbor, NY]. The final precipitate is dissolved in 70 ml of 20 mM Tris hydrochloride buffer, pH 8.0.

This DNA is reacted with 8-azidoethidium under the following conditions. The reaction mixture is prepared with 33 ml of 2.7 mg DNA/ml, 13.5 ml of 4.95 mM 8-azidoethidium, 13.5 ml of 0.2 M Tris-hydrochloride buffer, pH 8.0, 2 M NaCl, and 76 ml water. The mixture is placed in a 250 ml beaker with a water jacket maintained at 22°C. The mixture is stirred and illuminated for 60 minutes by a 150 watt spotlight at a distance of 10 cm. This photolysis is repeated with an identical reaction mixture.

The photolyzed reaction mixtures are combined and extracted 10-times with an equal volume each time of n-butanol saturated with 20 mM Tris-hydrochloride buffer, pH 8.0, 0.2 M NaCl. The extracted DNA solution is combined with 23 ml of 4.95 mM 8-azidoethidium and 77 ml of 20 mM Tris-hydrochloride buffer, pH 8.0, 0.2 M NaCl. This solution is stirred in the water-jacketed beaker and photolyzed for 90 minutes. The reaction products are extracted 10 times with buffer saturated butanol as

described above and the DNA is precipitated with ethanol. The precipitate is dissolved in 10 mM Tris-hydrochloride buffer, pH 8.0, 1 mM EDTA and the absorbances at 260 and 490 nm are recorded. Calculations made es described in Example 1A above indicate 1 ethidium residue is incorporated per 4.5 DNA base pairs.

Methylated thyroglobulin is prepared as follows: One hundred milligrams of bovine thyroglobulin (Sigma Chemical Co., St. Louis, MO) is combined with 10 ml of anhydrous methanol and 400 µl of 2.55 M HCl in methanol. This mixture is stirred on a rotary mixer at room temperature for 5 days. The precipitate is collected by centrifugation and washed twice with methanol and twice with ethanol. Then it is dried under vacuum overnight. About 82 mg of dry powder is obtained.

Antiserum to covalent ethidium-DNA is titered by an enzyme label immunosorbant assay. Polynucleotides are adsorbed onto the walls of polystyrene microtiter plates and then the rabbit antibody is allowed to bind. Finally the antibody is detected with peroxidase labeled goat anti-rabbit IgG.

Fifty microliter (50 µl) aliquots of solutions containing 5 µg of polynucleotide per ml in 15 mM sodium citrate, pH 7.0, 0.15 M NaCl is dispensed into wells of Immulon II microtiter plates (Dynatek, Alexandria, VA) and shaken gently at room temperature for 2 hours. Then the wells are emptied and washed with 10 mM sodium phosphate buffer, pH 7.4, 0.15 M NaCl, 0.5% bovine serum albumin and 0.5% Tween 20 (PBS/BSA/Tween$^{(R)}$).

Rabbit antiserum is diluted into 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl, 0.5% BSA and 50 µl aliquots are added to the wells and allowed to stand for 30 minutes. The wells are washed three times with PBS/BSA/Tween$^{(R)}$. Peroxidase covalently coupled to goat-antirabbit IgG (Cappel Laboratories, Cochranville, PA) is diluted 500-fold in 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl, 0.5% BSA and 50 µl aliquots are added

to each well. This solution is allowed to stand in the wells for 30 minutes at room temperature and then the wells are washed three times with PBS/BSA/Tween(R).

On hundred micromolar (100 μM) ethidium bromide is included in the diluted antiserum of wells containing noncovalent ethidium-DNA complex and the ethidium control wells. All wash solutions and reagents described above for processing these wells contain 100 μM ethidium.

A peroxidase substrate solution is prepared with:

20 mg o-phenylenediamine
5 ml 0.5 M $NaHPO_4$
12 ml 0.1 M sodium citrate
13 ml water
20 μl 30% hydrogen peroxide

Seventy-five microliters (75 μl) of substrate solution is added per well and allowed to react for 10 minutes at room temperature. The reactions are quenched by addition of 50 μl of 2.5 M sulfuric acid. Then the absorbances at 488 nm are recorded with a Artek Model 210 microliter plate photometer (Dynatek, Alexandria, VA).

Normal rabbit serum is used as a control and is processed as described for the rabbit antiserum

The results are given in Table A and show that antibody in the control rabbit serum does not bind at significant levels to any of the coated or uncoated wells. It might have a weak antibody titer to single stranded DNA.

The antiserum to the covalent ethidium-DNA has very high titer to the covalent ethidium-DNA. Part of these antibodies are probably binding to ethidium residues that are coupled covalently to the phosphate ribose chain. This conclusion is based on the observation that the titers to the noncovalent ethidium-DNA complex are much lower (see Table A).

These results demonstrate that antibodies can be raised to the ethidium-DNA intercalation complex which do not crossreact significantly with native single or double stranded nucleic acid.

Table A

Dilution	Absorbances (488 nm)					
Antiserum	Buffer Control	Covalent Ethidium-DNA	Double-strand DNA	Noncovalent Ethidium-DNA	Ethidium Control	Single-strand DNA
50	0.067	1.2	0.126	0.825	0.049	0.283
200	0.032	1.2	0.068	0.597	0.021	0.184
800	0.022	1.2	0.067	0.30	0.016	0.174
Control Serum						
50	0.038	0.053	0.091	0.031	0.023	0.245
200	0.025	0.044	0.082	0.016	0.017	0.181
800	0.017	0.034	0.054	0.015	0.016	0.190

Notes:

1) The buffer control does not contain DNA on the wells.
2) Double-stranded DNA contains calf thymus DNA on the wells.
3) Noncovalent ethidium-DNA has calf thymus double-stranded DNA on the wells and 100 μM ethidium in the reagent and wash solutions.
4) Ethidium control does not have DNA on the wells but has 100 μM ethidium in the reagent and wash solutions.
5) The single-stranded DNA has heat denatured calf thymus DNA coated on the wells.

It is understood that the specification and examples are illustrative, but not limiting to the present invention and that other embodiments within the spirit and scope of the invention will suggest themselves to those skilled in the art.

WHAT IS CLAIMED IS:

1. A nucleic acid detection probe comprising a hybridizable single stranded protion of nucleic acid connected with a non-hybridizable, single or double stranded nucleic acid portion, the non-hybridizable portion including a recognition site for binding by a particular protein.

2. A detection probe according to claim 1, characterized in that the hybridizable portion is continuous with one of the strands of the non-hybridizable portion.

3. A detection probe according to claim 1 or 2, characterized in that the non-hybridizable portion includes a recognition site for a protein selected from the group of lactose repressor, galactose repressor, lambda repressor, catabolite gene activator protein and Cro protein.

4. A detection probe according to any of claims 1 to 3, characterized in that the non-hybridizable portion has been modified to create the protein recognition site by contact or reaction with a modifier compound which introduces a unique antigenic determinant into the non-hybridizable portion, the modifier compound preferably being an intercalating agent, a platinium-containing ligand or salt.

5. A detection probe according to any of claims 1 to 4, characterized in that it includes the particular protein bound to the protein-specific site of the non-hybridizable portion, the protein preferably carrying a label selected from an enzymatically active group, a fluorescer, a chromophore, a luminescer, a

specifically bindable ligand, or a radioisotope.

6. A detection probe according to claim 5, characterized in that the protein is an antibody, or a fragment thereof.

7. A method for detecting a particular polynucleotide sequence in a test medium containing single stranded nucleic acids, characterized in that it comprises the steps of:

a) combining the test medium with a nucleic acid detection probe comprising at least one hybridizable single stranded base sequence which is substantially complementary to the sequence to be detected and a non-hybridizable double-stranded portion having a recognition site for binding by a particular protein, under conditions favorable to hybridization between the sequence to be detected and the complementary hybridizable sequence in the probe,

b) separating hybridized probe from unhybridized probe, and

c) adding to separated hybridized probe, the particular protein which binds the recognition site on the non-hybridizable portion of the probe and determining the protein which becomes bound to the solid support.

8. A method according to claim 7 which is a solid phase hybridization technique, wherein the single stranded nucleic acids from the test medium are immobilized on a solid support and wherein the protein associated with the solid support is determined or which is a

solid phase sandwich hybridization technique, wherein the test medium is combined with first and second nucleic acid probes each comprising at least one hybridizable single stranded base sequence which is substantially complementary to a mutually exclusive portion of the sequence to be detected and wherein one of the probes is immobilized on a solid support and the other has said non-hybridizable portion having said protein recognition site.

9. A reagent system for detecting a particular polynucleotide sequence in a test medium, comprising:

a) a nucleic acid detection probe according to any of claims 1 to 4, and

b) said particular protein capable of binding to said recognition site on the detection probe, the protein preferably carrying a label.

10. A reagent system according to claim 9, characterized in that the protein is an antibody, or a fragment thereof, labeled with an enzymatically active group, a fluorescer, a chromophore, a luminescer, a specifically bindable ligand, or a radio-isotope.

11. An antibody, or fragment thereof, capable of binding with an intercalation complex formed between double stranded nucleic acid and a nucleic acid, and preferably substantially incapable of binding to single stranded nucleic acids.

12. An antibody or fragment thereof according to claim 11, characterizedin that it is labeled with a detectable chemical group such as an enzymatically active group, a fluorescer, a chromophore, a luminescer, a specifically bindable ligand, or a radioisotope.

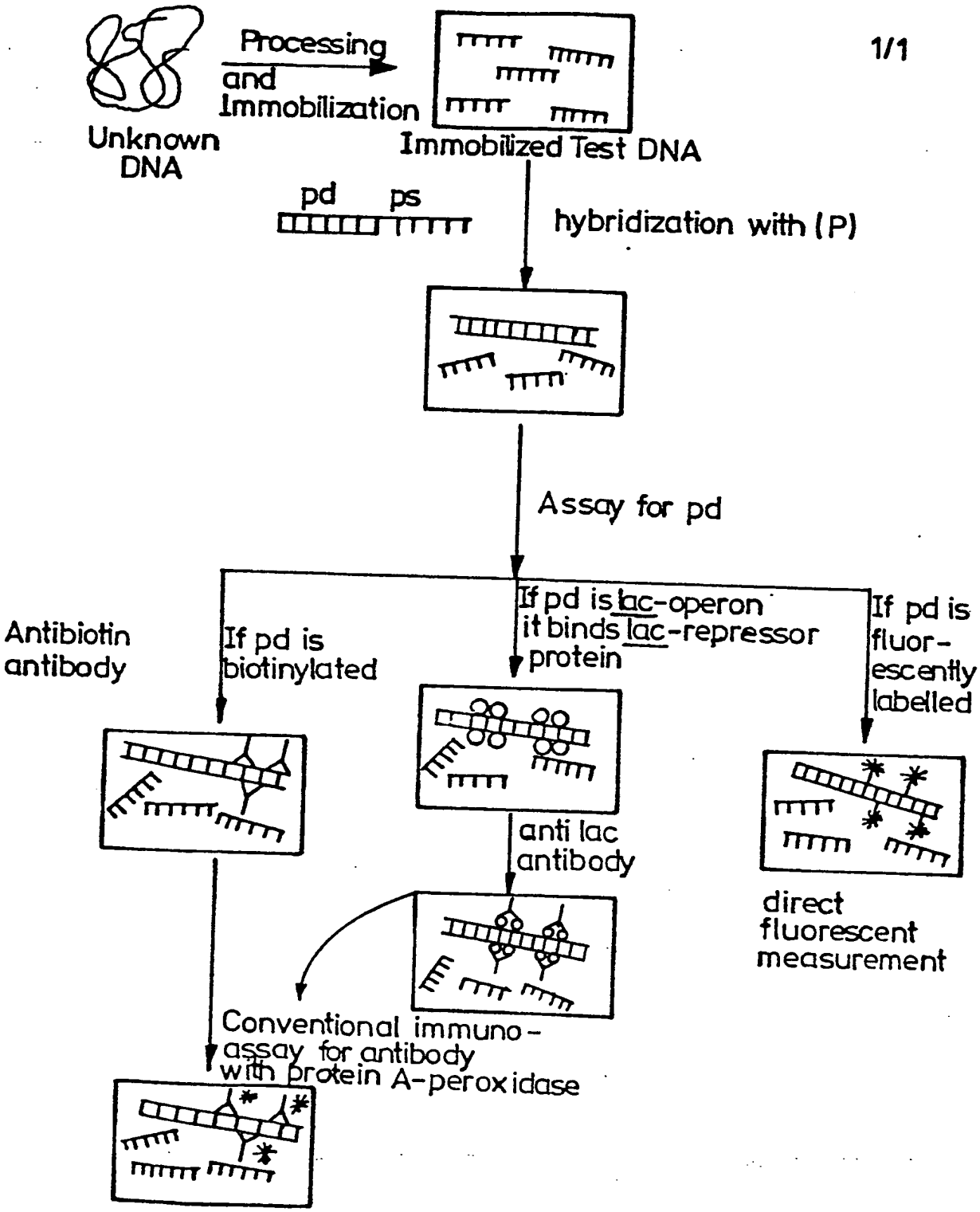
Processing
and
Immobilization
Unknown
DNA
Immobilized Test DNA
pd
ps
hybridization with (P)
Assay for pd
Antibiotin
antibody
If pd is
biotinylated
If pd is lac-operon
it binds lac-repressor
protein
If pd is
fluor-
escently
labelled
anti lac
antibody
direct
fluorescent
measurement
Conventional immuno-
assay for antibody
with protein A-peroxidase

FIG. 1

European Patent Office

EUROPEAN SEARCH REPORT

0147665
Application number

EP 84114536.0

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	EP - A1 - 0 079 139 (ORION CORPORATION, LTD.) * Abstract * --	1,7,9	G 01 N 33/50 G 01 N 33/531 C 12 Q 1/68
D,A	ANNUAL REVIEW OF BIOPHYSICS AND BIOENGINEERING, vol. 10, 1981 H.M. BERMAN, P.R. YOUNG "The Interaction of Intercalating Drugs with Nucleic Acids" pages 87-114 * Totality * --	1	
D,A	ACCOUNTS OF CHEMICAL RESEARCH, vol. 11, 1978 S.J. LIPPARD "Platinum Complexes: Probes of Polynucleotide Structure and Antitumor Drugs" pages 211-217 * Totality * ----	1,4	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

C 12 Q
G 01 N 33/00

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	20-03-1985	SCHNASS

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO Form 1503 03.82